Toxicon 54 (2009) 184–191

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Translocation of ricin across polarized human bronchial epithelial cells

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ARTICLE INFO

Article history:
Received 12 December 2008
Received in revised form 30 March 2009
Accepted 1 April 2009
Available online 15 April 2009

Keywords:
Ricin
Transcytosis
Translocation
Bronchial epithelial cells
Inhalational toxicity

ABSTRACT

Due to widespread availability, toxicity, and potential for use as a bioterrorism agent, ricin is classified as a category B select agent. While ricin can be internalized by a number of routes, inhalation is particularly problematic. The resulting damage leads to irreversible pulmonary edema and death. Our study describes a model system developed to investigate the effects of ricin on respiratory epithelium. Human bronchial epithelial (HBE) cells were cultured on collagen IV-coated inserts until polarized epithelial cell monolayers developed. Ricin was added to the apical or basal medium and damage to the cell monolayer was then assessed. Within a few hours after exposure, the cell monolayer was permeable to paracellular passage of the toxin. A mouse anti-ricin antibody neutralized ricin and prevented cellular damage as long as the antibody was present before the addition of toxin. These studies suggested that effective therapeutic agents or antibodies neutralizing ricin biological activity must be present at the apical surface of epithelial cells. The *in vitro* system developed here provides a method by which to screen potential therapeutics for protecting lung epithelial cells against ricin intoxication.

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1. Introduction

Native to tropical east Africa, castor bean plants (*Ricinus communis*) are commercially cultivated in many areas of the world for their valuable oil used in numerous products, such as soaps, cosmetics, medicines, motor oil, and nylon (Olsnes, 2004). Castor bean plants, grown for ornamental purposes, have also become weeds in many temperate areas of the world. In addition to the oil, castor beans contain ricin, a highly toxic protein (Bigalke and Rummel, 2005). A number of factors, such as widespread availability, ease of extraction, high toxicity, stability, and lack of specific antidotes, contribute to concerns about ricin being used as a biological threat agent. The ease with which aerosol dispersal of ricin could affect large populations justifies the United States Centers for Disease Control (CDC) placement of ricin as a category B agent (Khan et al., 2000).

Ricinus communis agglutinin II (ricin) belongs to the type 2 ribosome-inactivating protein family consisting of enzymatic and cell binding subunits linked by a disulfide bond. The enzymatic subunit, ricin A chain (RTA), excises a specific adenine residue in mammalian 28S rRNAs, the removal of which prevents elongation factor 2 from binding amino acids into a polypeptide chain, thereby inhibiting protein synthesis and causing cell death (Endo and Tsurugi, 1988; Nilsson and Nygard, 1986). By binding to galactoside residues present on cell-surface glycoproteins, the cell binding subunit, ricin B chain (RTB), enables ricin to be endocytosed. Once in the cell, the two chains separate and RTA is translocated into the cytosol, gaining access to ribosomes (Olsnes, 2004). Because ricin binds to many cell-surface receptors, almost all mammalian cell types are susceptible to ricin intoxication.

The symptoms and severity of ricin intoxication depend on the delivery route. Aerosolized ricin is considered a serious threat because it induces severe lung damage (Audi et al., 2005). Depending upon the dose, symptoms may include pulmonary edema and hypoxic respiratory

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0041-0101/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.toxicon.2009.04.003

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JAN 2010	**N/A**	**-**

4. TITLE AND SUBTITLE
Translocation of ricin across polarized human bronchial epithelial cells. Toxicon 54:184-191

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rushing, SR Saylor, ML Hale, ML

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

8. PERFORMING ORGANIZATION REPORT NUMBER
TR-08-109

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
Due to widespread availability, toxicity, and potential for use as a bioterrorism agent, ricin is classified as a category B select agent. While ricin can be internalized by a number of routes, inhaled ricin is particularly problematic. The resulting damage leads to irreversible pulmonary edema and death. Our study describes a model system developed to investigate the effects of ricin on repiratory epithelium. Human bronchial (HBE) cells werre cultured on collagedn IV inserts until polariazed epithelial cell monlayers developed. Ricin was added to the apical or basal medium and then damage to the cell monlayer was assessed. Within a few hours after exposure, ricin damaged HBE cells, rendering the cell monolayer permeable to the paracellular passage of ricin. A mouse anti-ricin antibody neutralized ricin and prevented cellular damage if the antibody was present before ricin bound to the epithelial cells. These studies suggested that, to be effective, therapeutic agents or antibodies neutralizing ricin biological activity must be present at the apical surface of epithelial cells. The in vitro system developed here provides a method by which to screen potential therapeutics for their ability to protect lung epithelial cells from ricin intoxication.

15. SUBJECT TERMS
ricin, translocation to cytosol, polarized bronchial cells, human cell culture, transcytosis, inhalation toxicity

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**8**	

failure, with fever, cough, dyspnea, nausea, chest tightness, cyanosis, hypotension, and circulatory collapse (Audi et al., 2005; Bigalke and Rummel, 2005). Because there is no antidote for ricin intoxication, treatment of victims is strictly palliative. Death usually ensues within 3–5 days, although patients who survive this critical initial period have a good prognosis (Bigalke and Rummel, 2005).

In order to develop effective drug therapies for ricin intoxication, identifying routes of intoxication becomes important, particularly since the toxin binds to and then kills most human cells. In the case of aerosolization, the first cells encountered are in the respiratory epithelium that forms a protective barrier over the underlying tissues. Because a lethal dose of ricin instilled via the pulmonary system may lead to systemic distribution with damage to the kidneys and other organs, characterizing how it crosses the epithelial cell barrier becomes a critical issue (Wong et al., 2007).

One method by which ricin might cross the epithelial cell barrier is by transcytosis, a process by which proteins cross from one side of the cell to the other as membrane-bound cargo (Tuma and Hubbard, 2003). Ricin has been shown to cross polarized Madin–Darby canine kidney (MDCK) and the human colon adenocarcinoma epithelial (Caco-2) cell monolayers via transcytosis, suggesting that this process may also be used by the toxin to cross lung epithelia (Brandli et al., 1990; Jackman et al., 1994). Therefore, an *in vitro* model was developed to determine if ricin uses transcytosis to cross the lung epithelial cell barrier. Unlike previous investigations with kidney and intestinal epithelial cells, our studies indicated that transcytosis did not play an important role in moving ricin from the lung into the vascular system. Rather, damage to the tight gap junctions permitted toxin to move around the cells, thus gaining entry by paracellular diffusion.

2. Materials and methods

2.1. Materials

Ricinus communis agglutinin II (ricin) was purchased from Vector Laboratories, Burlingame, CA. Mouse anti-deglycosylated ricin toxin A chain antibody (anti-dgRTA IgG) was prepared by PerImmune, Inc. (Rockville, MD) under contract for United States Army Medical Research Institute of Infectious Diseases, Frederick, MD (Lindsey et al., 2007). Alexa Fluor 680 antibody kits, a 10,000 MW dextran conjugated to Alexa 680 (dextran-680), rabbit anti-ZO-1 IgG, goat anti-rabbit IgG conjugated to Alexa 488, Texas Red phalloidin, Hoechst 33342 dye, polyacrylamide gel electrophoresis (PAGE) supplies, Earle's minimal essential medium (EMEM), RPMI-1640 medium, Hank's balanced salt solution (HBSS), L-glutamine (200 mM), non-essential amino acid (10 mM) solution, and penicillin–streptomycin liquid (10,000 units penicillin; 10,000 μg streptomycin) were obtained from Invitrogen, Carlsbad, CA. Bronchial epithelial growth medium (BEGM) and trypsin ReagentPak were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Hyclone, Logan, UT, and heat inactivated (56 °C, 30 min) before use. Cell culture plates and flasks were obtained from Corning Life Sciences, Corning, NY. BD Biocoat collagen type IV-coated 24-well plate inserts (1.0-μm pores) were purchased from BD Biosciences, Bedford, MA, while the Nunc Anopore membranes (0.02-μm pores) were from Nalge Nunc, Rochester, NY. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (CellTiter 96AQ) was purchased from Promega Corporation, Madison, WI. A bicinchoninic acid-based (BCA) kit to measure protein concentrations was purchased from Thermo Fisher Scientific, Inc., Rockford, IL. Immunoglobulin and protease free bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch, West Grove, PA and chemicals from Sigma Aldrich Chemicals, St. Louis, MO. Molecular weight markers for PAGE and Coomassie Blue protein stains were purchased from LI-COR, Inc., Lincoln, NE.

2.2. Labeling ricin with Alexa 680

To follow ricin translocation across epithelial cell monolayers, ricin was conjugated to a dye that fluoresces in the near infrared (NIR) range (Rushing et al., 2007). Ricin (1.5 mg) was suspended in 0.5 ml of phosphate-buffered saline (PBS), pH 7.2, and labeled with the Alexa Fluor 680 NIR dye according to the manufacturer's instructions. Final concentrations of labeled proteins were measured by the BCA protein assay. SDS-PAGE gel electrophoresis conducted under non-reducing conditions revealed a single labeled band of approximately 60 kDa when scanned in the 700 nm channel of the Odyssey imaging system (LI-COR, Inc.). The gel, subsequently stained with Coomassie Blue protein stain, showed that both unlabeled and labeled ricin (1 μg/ml) had a 60 kDa band of similar density. When assessed using Jurkat cells, both the NIR-labeled ricin (ricin-680) and the unlabeled ricin standard exhibited similar cytotoxicity, indicating that labeling had no effect upon biological activity (data not shown).

2.3. Cell culture

The 16HBE14o− human bronchial epithelial cell line (HBE) was obtained from Dr D.C. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA. HBE cells were maintained in EMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acid solution, penicillin (50 units/ml), and streptomycin (50 μg/ml). When the monolayer reached approximately 80% confluency, cells were trypsinized using the ReagentPak, and then subcultured in 175 cm^2 tissue flasks with cells for experiments between passages 4 and 16. The Jurkat human T lymphocyte leukemia cell line (American Type Culture Collection, Manassas, VA) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (RPMI-FBS), and passed twice weekly. All cells were maintained in a 37 °C incubator with 5% CO_2.

2.4. Ricin cytotoxicity assay

Ricin biological activity was determined using a Jurkat cell viability assay in which Jurkat cells were resuspended in RPMI-FBS at a density of 2×10^6 cells/ml and transferred to a 96-well plate (100 μl/well). Samples (50 μl) were then

added to the wells and incubated (37 °C, 5% CO_2) for 24 h before the addition of CellTiter 96AQ (20 μl). Plates were incubated for one additional hour and absorbance was then measured at 490 nm (A_{490}) on a Victor multi-plate reader (Perkin-Elmer, Waltham, MA). Ricin dilutions (toxin standard) were included in each assay. Cell viability was expressed as the % Control = [(Sample A_{490}/Control A_{490}) × 100].

2.5. Transport assay

Collagen IV-coated, 24-well cell culture inserts were prepared according to the vendor's directions. HBE cells (5.0×10^5 HBE cells in 500 μl BEGM) were added to each insert (apical chamber) and placed in wells containing 500 μl of BEGM (basal chamber). BEGM was replaced on days 3, 5, and 7. Formation of contiguous cell monolayers was evaluated by microscopic examination following medium replacement. Development of tight gap junctions was monitored by measuring the transepithelial electrical resistance (TEER) using an EVOM epithelial volt ohmmeter (World Precision Instruments, Inc., Sarasota, FL).

When the TEER reached 600–1000 Ω/cm^2, 500 μl of BEGM containing dextran-680 (2-μg/ml) replaced the medium in the apical chamber to detect cell monolayer permeability (Amieva and Vogelmann, 2004). The following day, inserts were transferred to another 24-well plate and the basal medium scanned using the Odyssey imaging system. Presence of dextran-680 in basal medium indicated that the cell monolayer was not contiguous and those inserts were not used. Inserts with contiguous cell monolayers were then rinsed with HBSS to remove dextran-680, and fresh medium (500 μl) was added to the apical chambers.

For ricin treatments, medium in the apical or basal chamber was replaced with BEGM containing ricin-680. Aliquots (100 μl) of medium were removed from the basal and apical chambers at various time points and stored at −70 °C. In order to quantify the amount of toxin present, stored samples were thawed, transferred to a 96-well black, clear-bottom microtiter plate and scanned on the Odyssey imaging system. Ricin in the basal medium was calculated using a standard curve scanned in parallel with the samples. In some experiments, inserts were removed from the plate and the plate scanned directly. When the 24-well plate was scanned directly, a standard curve was included in one row of wells. Background fluorescence from wells without ricin was subtracted from the fluorescence value of each sample and standard. Ricin concentrations (ng/ml) were calculated from the standard curve and p values determined by an unpaired Student's t-test. Results were considered significant at $p < 0.05$.

2.6. Antibody neutralization assay

To determine whether anti-ricin antibodies prevented damage to the polarized HBE cell monolayer, 500 μl of BEGM containing 4.9 μg anti-dgRTA IgG was added to the basal chamber and the plate incubated 3 h before adding ricin-680 to the apical medium. The incubation step provided time for the antibodies to cross the cell monolayer (Spiekermann et al., 2002). For other wells, ricin-680 (250 ng) and anti-dgRTA IgG (4.9 μg) were added to 500 μl BEGM and incubated 40 min before replacing apical medium of inserts. At 21 and 48 h, inserts were transferred to another plate for temporary storage while the plate was scanned using the Odyssey imaging system. TEER measurements were performed and the inserts returned to the experimental plate. At 48 h, basal medium aliquots were tested for ricin biological activity using the Jurkat cytotoxicity assay.

2.7. Confocal microscopy sample preparation

All solutions used in the staining procedure were prepared in phosphate-buffered saline (PBS), pH 7.4. Polarized HBE cell monolayers were cultured in 0.02-μm Anopore cell culture inserts. After 7 days in culture, medium containing ricin, ricin and anti-dgRTA IgG, or medium alone was added to the cell monolayers and incubated for 21 h. Cell monolayers in the inserts were fixed (30 min, 25 °C) in pre-warmed 3.7% formaldehyde solution. Monolayers were rinsed (5 min) with PBS, and further permeabilization of plasma membranes done by adding Triton X-100 (0.02%). After 15 min (25 °C), a 2% BSA solution was added to the monolayers to block non-specific binding sites. Rabbit anti-ZO-1 IgG, diluted in 1% BSA solution (1% BSA), was then added and incubated for 1 h (37 °C). Monolayers were washed three times (5 min each) in PBS and counterstained with goat anti-rabbit IgG–Alexa 488, diluted in 1% BSA (1 h, 37 °C). Texas Red phalloidin (1:25 dilution) was then added for visualization of actin filaments. After a 5-min wash in PBS, Hoechst dye 33342 was added (15 min, 25 °C) to visualize the nucleus. After labeling, monolayers were washed twice in PBS and once in distilled water. The Anopore membranes were removed from the inserts, mounted on microscope slides (Daigger, Vernon Hill, IL) with Fluoromount G mounting medium (Fisher Scientific, Suwanee, GA) and covered with a round glass cover slip (Fisher Scientific).

3. Results

3.1. Characterization of ricin transport in HBE cells

Ricin has been reported to undergo transcytosis across MDCK and Caco-2 polarized cell monolayers in both the apical-to-basal and basal-to-apical directions (Jackman et al., 1994; van Deurs et al., 1990). An *in vitro* model using the human bronchial epithelial cell line 16HBE14o− was established to investigate whether ricin crossed lung epithelial cells by transcytosis or by paracellular transport. To measure the amount of ricin that crossed the monolayer, our model used ricin labeled with an NIR dye, thereby avoiding the use and disposal problems associated with using radioisotopes (Rushing et al., 2007). Unlike an ELISA, detecting NIR fluorescence is rapid and performed in real-time. The dye was not cytotoxic to HBE cells and did not affect cell monolayer integrity (data not shown). In addition, labeling the toxin with the NIR dye did not affect toxin biological activity, making the ricin-680 suitable for investigating efficacy of therapeutic agents, as well as

studying the basic biology of ricin exposure in the HBE cells.

Transport of the toxin across HBE cell monolayers was evaluated in both the apical-to-basal and basal-to-apical directions. Over time, there was a gradual increase in the amount of ricin that translocated across the monolayer although the amount varied among the different treatment groups (Table 1). When assessed at 1.25 and 2.25 h in the apical-to-basal direction, ricin crossing the monolayers was similar in both the transiently exposed samples and the continuously exposed samples. By 4.25 h, however, the amount transported was significantly greater in the continuously exposed (7.9 ± 0.5 ng/ml) than in the transiently exposed (6.2 ± 1.1 ng/ml) samples. After 20 h, the amount from the continuously exposed samples was four-fold greater than the transiently exposed samples. In the basal-to-apical direction, significantly more ricin translocated when continuously present as compared to the transiently exposed samples. The large decrease in ricin moving across the monolayer when transiently applied as compared to when it was continuously present, suggested that the toxin translocated via a passive mechanism.

3.2. Exposure to ricin results in monolayer permeability

Initial experiments showed that ricin crossed the HBE cell monolayer, but did not confirm whether the toxin crossed the monolayer by transcytosis or by paracellular movement. Therefore, TEER measurements were performed to determine whether ricin affected cell monolayer integrity. Polarized HBE cell monolayers were treated with various dilutions of ricin and TEERs measured at 0, 4, and 28 h (Fig. 1A). At the beginning of the experiment, all measurements ranged between 780 and 980 Ω/cm^2. Four hours later, TEERs in all samples had decreased slightly, ranging between 700 and 800 Ω/cm^2. After 28 h, TEER measurements in the ricin-treated groups had fallen and were significantly different from the TEER of the untreated control. Although the TEER of the control sample dropped slightly from 0 h (780 Ω/cm^2) to 28 h (660 Ω/cm^2), it remained significantly higher than in the ricin-treated cell monolayers (all between 90 and 160 Ω/cm^2).

Other investigators have noted that while TEER measurements provide a method to monitor development of tight gap junctions, TEERs may not provide the sensitivity needed to confirm the monolayer's integrity (Tuma and Hubbard, 2003). Therefore additional assessment was used to confirm cell monolayer integrity. Dextran-680, a high molecular weight polysaccharide, does not cross epithelial cells by transcytosis and can be used to follow the development of polarized cell monolayers (Amieva and Vogelmann, 2004). Since the amount of dextran-680 detected in the basal medium will also reflect the degree to which tight gap junctions among cells have been lost (i.e., cell damage), dextran-680 was used to follow

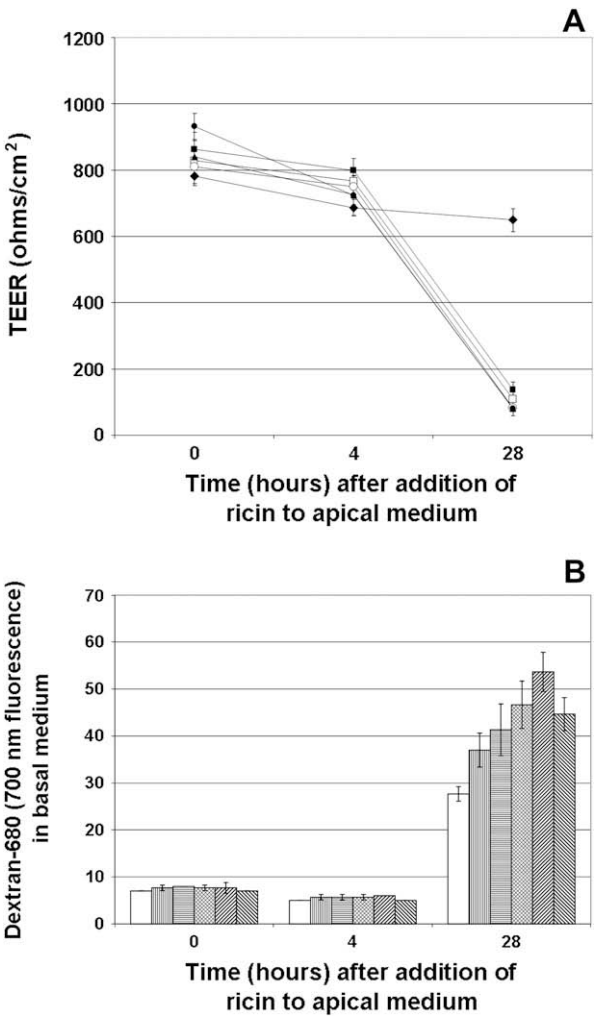

Fig. 1. Damage to HBE cell monolayer integrity exposed to various concentrations of ricin in the apical medium was followed by measuring (A) the transepithelial electrical resistance (TEER) or (B) the permeability by measuring the amount of dextran-680 detected in the basal medium after addition of the toxin. (A) For TEER measurements, solid diamonds represent the untreated control (no ricin) while ricin concentrations are represented as follows: solid square, 0.2 ng/ml; open square, 2.5 ng/ml; open circle, 25 ng/ml; closed triangle, 250 ng/ml; and closed circle, 2500 ng/ml. (B) For dextran-680 measurements, white bars represent the untreated control (no ricin) and ricin concentrations are represented as follows: vertical lines, 0.2 ng/ml; horizontal lines, 2.5 ng/ml; diamonds, 25 ng/ml; left slanted lines, 250 ng/ml; and right slanted lines, 2500 ng/ml. The asterisks indicate permeability is significantly different from the controls at the same time point, as determined by the Student's *t*-test. Data represented the average ± standard deviation for three inserts.

Table 1
Transport of ricin across polarized HBE cell monolayers.

Direction of transport	Time of sample collection[a]			
	1.25 h	2.25 h	4.25 h	20 h
Apical to basal	1.1 ± 1.2	5.3 ± 0.4	**7.9 ± 0.5**[c]	**48.8 ± 4.2**
Apical to basal[b]	3.5 ± 2.9	6.4 ± 1.3	**6.2 ± 1.1**	**11.7 ± 1.3**
Basal to apical	**3.7 ± 0.9**	**4.3 ± 0.6**	**7.3 ± 0.9**	**53.1 ± 16.6**
Basal to apical[b]	**1.8 ± 0.7**	**2.0 ± 0.5**	**2.3 ± 0.8**	**4.8 ± 1.7**

[a] All samples were initially treated with 500 ng/ml of ricin. Average values and standard deviations for each treatment group ($n = 4$) are displayed in ng/ml of ricin.
[b] Ricin was removed from these samples 1 h after its application.
[c] Within each direction of transport, continuously and transiently exposed samples that were significantly ($p < 0.05$) different at the same time point are indicated by bold numbers.

cell monolayer damage after ricin treatment (Rushing et al., 2007).

When cell monolayer permeability was measured at 0 and 4 h, no differences among the five treatment groups and untreated controls were detected (Fig. 1B). By 28 h when compared to the untreated control, all ricin concentrations showed a significant increase in the amount of dextran-680 detected in the basal medium. At 28 h, untreated control monolayers showed an increase in permeability when compared to the 0 and 4 h time points, but the increase was significantly less than that detected in ricin-treated monolayers. While TEER measurements (Fig. 1A) of the untreated controls decreased slightly over time, differences were more subtle indicating that the TEER was not as sensitive a method for measuring the integrity of the cell monolayer. Importantly, results of these experiments indicated that even small doses of ricin could affect epithelial cell monolayer integrity, a point that should be taken into consideration during development of therapeutics against ricin intoxication.

3.3. Ricin is neutralized by anti-dgRTA antibody but protection of HBE cells from ricin-induced damage depended on the site

To demonstrate the effectiveness of this *in vitro* assay for evaluating potential ricin therapeutics, neutralization of the toxin by polyclonal anti-dgRTA IgG was examined. Since antibodies elicited by a ricin vaccine would enter the circulatory system and reach the airways by crossing the lung epithelium from the basolateral side, anti-dgRTA IgG was also added to the basal medium 3 h before adding ricin to the apical medium. Twenty-four and 48 h later, TEERs were measured and compared to their pretreatment values (Fig. 2A). While TEERs of untreated controls did not change significantly, TEERs of cell monolayers treated with ricin alone decreased after 21 h ($27 \pm 3\%$) and continued to decrease ($3 \pm 0\%$ by 48 h), TEER measurements of cell monolayers incubated with antibody in the basal medium before adding toxin to the apical side (basal anti-dgRTA IgG) were similar to those of cell monolayers treated with ricin alone (21 h, $31 \pm 6\%$ and 48 h, $4 \pm 1\%$). By contrast, at 21 h, samples in which ricin was pre-incubated with anti-dgRTA IgG before being added to the apical chamber (apical anti-dgRTA IgG), the TEER was the same as pretreatment values ($91 \pm 13\%$). By 48 h, however, the TEER had dropped ($12 \pm 2\%$), demonstrating delayed damage to the monolayer.

In conjunction with measuring TEERs, plates were scanned (without inserts) at 21 and 48 h, and toxin concentration in the basal medium calculated using a standard curve (Fig. 2B). There was little difference between the amount of ricin present in the ricin alone (9 ± 0.3 ng/ml) and basal anti-dgRTA IgG (10 ± 2.6 ng/ml) samples. However, the amount of ricin (3.5 ± 0.2 ng/ml) was significantly reduced in the apical anti-dgRTA samples. As indicated by the higher TEER (Fig. 2A), the integrity of the monolayer was not damaged. Because IgG readily crosses polarized epithelial cells by transcytosis, some ricin bound to anti-dgRTA IgG could have been taken across the cells by transcytosis. Transcytosis is not an efficient process

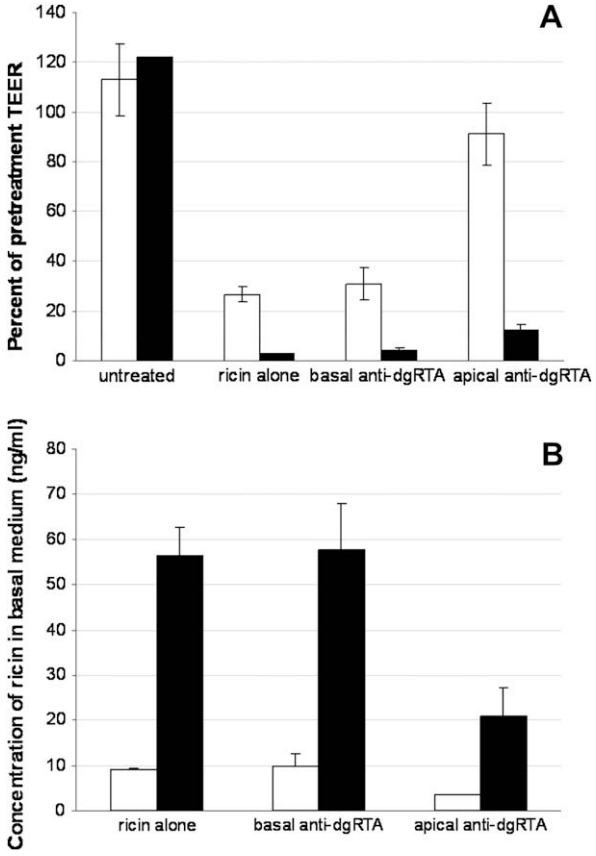

Fig. 2. Neutralization of ricin by anti-ricin antibodies. Ricin biological activity was neutralized by adding anti-dgRTA IgG (4.9 μg) to the basal chamber 3 h before the addition of ricin (250 ng) to the apical media (basal anti-dgRTA IgG); or the anti-dgRTA IgG (4.9 μg) was incubated with ricin-680 (250 ng) 40 min before being added to the apical side of the insert (apical anti-dgRTA IgG). Controls in each experiment included an untreated control (no ricin) and ricin alone. White bars are measurements taken at 21 h and black bars measurements at 48 h. (A) TEERs at each time point were measured and expressed as a percentage of the initial pretreatment value. (B) The fluorescence of the basal medium was measured and the amount of ricin transported into the basal chamber was calculated using a ricin standard curve. Data represent the average ± standard deviation for two inserts.

and could account for the reduced amount of ricin (Spiekermann et al., 2002; Tuma and Hubbard, 2003).

By 48 h, the amount of toxin in the basal medium of all samples increased significantly. Ricin alone and the basal anti-dgRTA IgG samples contained significantly more toxin (56.4 ± 6.2 and 57.6 ± 10.4 ng/ml, respectively) than the apical anti-dgRTA IgG samples (20.9 ± 6.5 ng/ml). The higher TEER and reduced amount of ricin detected in the basal medium suggested that damage to the cell monolayer was mitigated, thus reducing paracellular transport in those samples where ricin was pre-incubated with anti-ricin antibodies.

Experiments were then performed to determine whether ricin's biological activity had been neutralized by anti-dgRTA IgG (Table 2). At the end of the experiment (48 h), aliquots of the basal medium were added to Jurkat cells and cytotoxicity assessed by comparing treated

Table 2
Detection of biologically active ricin in the basal medium.

Basal medium from inserts added to Jurkat cells	Percent (%) survival of Jurkat cells[a]	p value[b]
Without IgG[c]	26.2 ± 0.4	
Basal anti-dgRTA IgG[d]	105.5 ± 2.1	*
Apical anti-dgRTA IgG[e]	98.8 ± 1.2	*

* Significant ($p < 0.05$).
[a] Percent (%) survival compared to control (no treatment) cells. Average values and standard deviations for each treatment group ($n = 3$).
[b] p value is based on survival in medium without IgG.
[c] All samples contained ricin added to apical medium.
[d] Anti-dgRTA IgG added to basal medium 3 h before ricin was added to apical medium.
[e] Anti-dgRTA IgG incubated with ricin before being added to apical medium.

samples with untreated controls. The low survival rate of Jurkat cells incubated with medium from samples containing ricin alone confirmed that the toxin remained biologically active. In both basal and apical anti-dgRTA IgG samples, ricin biological activity was neutralized as cell survival rates were similar to those of the untreated control. While basal medium from the apical anti-dgRTA samples contained less ricin than other samples (Fig. 2B), the quantity was above the effective dose required to kill 100% of the cells (10 ng/ml, data not shown). As indicated by the drop in TEERs, when gaps developed in the cell monolayer, antibody and antibody complexed to the toxin could cross the membrane by paracellular transport. Thus the low toxicity observed in the basal medium from apical dgRTA samples could be attributed to the presence of neutralizing anti-dgRTA IgG in the basal medium.

3.4. Neutralization of ricin with anti-ricin antibody reduces HBE cell monolayer damage

HBE cell monolayers, grown on optically transparent Anopore membranes, were treated with ricin and anti-dgRTA IgG and then stained to visualize DNA, actin, and ZO-1 for morphological analysis using confocal microscopy. The untreated control monolayer (Fig. 3A) was characterized by densely packed cells with ovoid nuclei (blue). In most cells, a narrow layer of cytoplasm surrounded an intact nucleus. Actin (red) had a diffuse cytoplasmic distribution, but also strongly co-localized along the cell periphery. The anti-ZO-1 IgG recognizes a protein localized in tight gap junctions. ZO-1 staining (green) surrounding most cells indicated tight gap junction formation. In contrast, the cells exposed to ricin exhibited pronounced morphological changes (Fig. 3B). Most cells appeared larger and nuclear material was fragmented and amorphous, with few remaining ovoid nuclei. Actin formed randomly dispersed clumps that no longer co-localized with the cell periphery. Although the cells were still contiguous, ZO-1 staining along the cell–cell junctions was attenuated and unevenly distributed, indicating damage to the tight gap junctions.

When HBE cell monolayers were treated with anti-dgRTA IgG, antibody added in the basal medium had limited effect on neutralizing ricin biological activity (Fig. 3C). Although IgG has been shown to cross epithelial cells by transcytosis (Tuma and Hubbard, 2003), evidently there was insufficient IgG in the apical medium to abrogate ricin toxicity. Similar to monolayers treated with ricin, the cells were larger, although not to the same extent. Actin appeared clumped and not associated with the cell periphery while nuclei in many cells were fragmented. Tight gap junctions appeared to be compromised as shown by uneven ZO-1 staining. Conversely, pre-incubating ricin with anti-dgRTA IgG before adding to the apical side of the monolayer showed little morphological change (Fig. 3D) and were similar to untreated controls. Actin associated with ZO-1 and nuclei were mostly ovoid with little fragmentation. Taken together, this study confirmed our previous observations that the anti-dgRTA IgG neutralized ricin more effectively when applied to the apical side of the monolayer.

4. Discussion

Although there is concern over the use of aerosolized ricin as a biowarfare weapon, there are no specific therapies available to treat ricin intoxication. Since ricin has been shown to cause severe damage to the lung, understanding toxicity mechanisms in cells of the respiratory system is crucial for successful development of specific therapeutics. A previous study demonstrated that the size of aerosolized ricin particles is a significant factor affecting toxicity and internal deposition of inhaled ricin in mice (Roy et al., 2003). Larger particles (median diameter of 5 μm) deposited preferentially in the trachea, with fewer than 20% penetrating deeper into the lungs. Smaller particles (median diameter of 1 μm) penetrated into the bronchi and some alveoli causing pronounced damage to the bronchial epithelium. All mice exposed to small particles died within 72 h post-exposure.

Since bronchial passages are primary tissues affected by inhaled ricin, a human bronchial cell line was selected for an *in vitro* system (Cozens et al., 1994). The need to investigate ricin effects on human bronchial epithelial cells becomes evident as previous reports suggest dramatic differences between mouse and human lungs after toxin exposure (Baluna et al., 1999; Lindstrom et al., 1997).

Ricin transport across epithelial cells has been investigated extensively in MDCK cells and is shown to occur by transcytosis (Brandli et al., 1990; Jackman et al., 1994; Stirpe, 2004). Whether the toxin utilizes transcytotic pathways to cross lung epithelial cells had not been investigated. Although much of the damage to the respiratory system results from direct contact between ricin and epithelial cells, the danger that ricin poses to endothelial or other tissues depends both upon ricin's access to those tissues and upon toxin stability *in vivo* (Wong et al., 2007). After aerosol exposure, ricin is mostly localized to the respiratory and gastrointestinal tracts; however, after high doses, there is evidence that the toxin can enter other organs via the circulatory system (Doebler et al., 1995; Wong et al., 2007).

Determining how the toxin crosses the epithelial cell barrier, whether by transcytosis or by paracellular transport, is crucial for gaining insight into the pathogenesis of inhaled ricin. For example, less ricin would be expected to cross epithelial cells via transcytosis than by paracellular

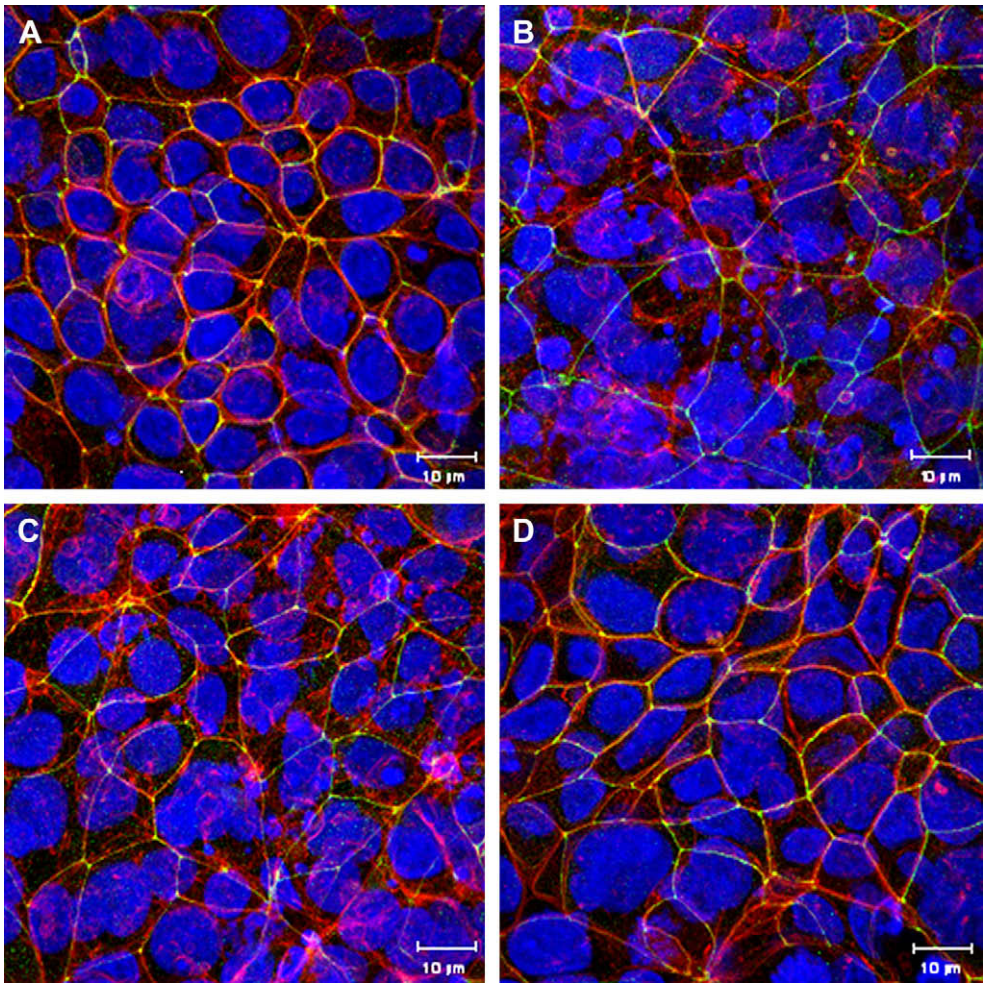

Fig. 3. Confocal microscopy of HBE cells exposed to ricin. Cells were grown on Anopore membranes until polarized cell monolayers developed. The monolayers were then subjected to the following treatments: (A) untreated (control); (B) ricin alone; (C) anti-dgRTA IgG in the basal medium 3 h before the addition of ricin to the apical medium; or (D) ricin pre-incubated with anti-dgRTA IgG before adding to the apical medium. After 21 h, the monolayers were fixed and stained to visualize nuclear DNA, with Hoechst 33342 (blue); actin, with Texas Red phalloidin (red); and tight gap junctions, with anti-ZO-1 followed by an Alexa Fluor 488 labeled secondary antibody (green).

transport (Brandli et al., 1990; Jackman et al., 1994; Stirpe, 2004). Passage by paracellular routes could therefore permit more toxin to enter the vascular endothelium, leading to greater tissue damage.

In our studies, ricin-induced damage to epithelial cell monolayers became evident several hours post-intoxication. A drop in TEER and loss of monolayer impermeability were accompanied by an increased rate of toxin transport across the cell monolayer. Damage to the monolayer occurred whether ricin exposure was transient or continuous. While this study could not rule out the possibility that the toxin utilized transcytosis, transport that occurred independent of damage to the monolayer, even at low ($<$1 ng/ml) toxin concentrations, was not detected. Our previous studies investigating staphylococcal enterotoxin B transcytosis across HBE cell monolayers confirmed that the NIR method provides an accurate measurement of transcytosis (Rushing et al., 2007). Altogether, our studies indicated that translocation of ricin occurred chiefly by paracellular routes.

As suggested by morphological examination of ricin-treated HBE cell monolayers, several factors may contribute to the cell damage. For example, ricin triggers cell death by apoptosis in a number of cell lines, and apoptotic cells would be expected to dissociate from the monolayer (Korcheva et al., 2007; Rao et al., 2005; Wu et al., 2004). Microscopic examination of toxin-treated HBE cells revealed nuclear fragmentation, a hallmark of apoptosis. However, apoptosis did not appear to be the universal response as not all cells had fragmented nuclei or other markers of apoptosis (e.g., cell shrinkage and membrane blebbing). Further studies are under way to determine if other death pathways occur in these cells after ricin exposure.

A significant observation of HBE cell monolayers treated with ricin was a pronounced change in morphology. Instead of the tightly packed and compact morphology of untreated cells, intoxication resulted in a spread-out squamous cell-like appearance. This is similar to the behavior of bronchial epithelial cells in mice, in which lung injury induced by exposure to naphthalene, specifically targets and kills Clara

cells, the non-ciliated secretory cells of the bronchial epithelium (Lawson et al., 2002; Stripp and Reynolds, 2008). After the lungs are exposed to the chemical, bronchial epithelial cells develop a more squamous cell-like morphology in order to fill in the gaps left by necrotic Clara cells that have detached from the basement membrane. By filling in these gaps, the protective epithelial barrier remains intact while the injured sites are being repaired (Lawson et al., 2002). Although in our studies, HBE cells underwent similar morphological changes, the monolayer did not maintain an impermeable barrier, as evidenced by both the drop in TEER and disruption of tight junctions between cells.

Our study revealed that a complex series of events occurred at the cellular level after exposure to ricin. Clearance of ricin was a gradual process, becoming less efficient as the tissue damage progresses. A clear implication of this work was that a specific anti-ricin therapy would be more successful if implemented quickly, as even a short exposure to ricin was sufficient to cause damage. Additionally, this study indicated that an important criterion for vaccines designed to protect against inhalation exposure must provide the apical surface of lung epithelia with antibody capable of neutralizing the toxin.

In previous studies, rats vaccinated with deglycosylated ricin A chain (dgRTA) developed less lung damage than rats vaccinated with a ricin toxoid. When bronchoalveolar lavage (BAL) collected from vaccinated rats was examined, there were significantly higher amounts of anti-ricin antibodies in animals vaccinated with dgRTA than those vaccinated with the ricin toxoid (Hale, data not published). Because the vaccines were administered intramuscularly, anti-ricin antibodies in the lung were primarily acquired by transcytosis from the basolateral epithelium to the apical side of alveolar sacs.

Regardless of the origin of antibodies in the lung, our *in vitro* system using human polarized lung epithelial cell monolayers provides a simple method to screen therapeutics for potential use against an aerosolized delivery of ricin. Additionally, our studies provided further morphologic evaluation of ricin intoxication in lung epithelial cells. Further studies are currently under way in our laboratory to refine and expand our *in vitro* model.

Acknowledgments

We are very grateful to Dr. Gordon Ruthel for technical advice and for providing the micrographs of our samples. This work was funded by DTRA Research Plan #133249. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by The United States Army.

Conflict of interest

The authors declare that there are no conflicts of interest.

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